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ARTICLE

Efficacy of an Adeno-associated Virus 8-Pseudotyped Vector in Glycogen Storage Disease Type II

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Glycogen storage disease type II (GSD-II; Pompe disease) causes death in infancy from cardiorespiratory failure. The underlying deficiency of acid α-glucosidase (GAA; acid maltase) can be corrected by liver-targeted gene therapy in GSD-II, if secretion of GAA is accompanied by receptor-mediated uptake in cardiac and skeletal muscle. An adeno-associated virus (AAV) vector encoding human (h) GAA was pseudotyped as AAV8 (AAV2/8) and injected intravenously into immunodeficient GSD-II mice. High levels of hGAA were maintained in plasma for 24 weeks following AAV2/8 vector administration. A marked increase in vector copy number in the liver was demonstrated for the AAV2/8 vector compared to the analogous AAV2/2 vector. GAA deficiency in the heart and skeletal muscle was corrected with the AAV2/8 vector in male GSD-II mice, consistent with receptor-mediated uptake of hGAA. Male GSD-II mice demonstrated complete correction of glycogen storage in heart and diaphragm with the AAV2/8 vector, while female GSD-II mice had correction only in the heart. A biomarker for GSD-II was reduced in both sexes following AAV2/8 vector administration. Therefore, GAA production with an AAV2/8 vector in a depot organ, the liver, generated evidence for efficacious gene therapy in a mouse model for GSD-II.

INTRODUCTION

Adeno-associated virus (AAV) vectors have several advantages for gene therapy in genetic disease, including persistent gene expression, lack of immune response to transduced cells, and no association of AAV with any human disease [1]. The cloning of alternative serotypes of AAV has improved the tropism of AAV vectors for specific target tissues by cross-packaging AAV2-based vectors with non-AAV2 capsids [2–5]. Specifically, if AAV2 vector genomes were cross-packaged as AAV1 (AAV2/1) and AAV5 (AAV2/5), muscle and liver were transduced more efficiently than with the original AAV2 vector [4]. For instance, AAV2/1 vectors encoding canine coagulation factor IX (FIX) produced FIX in skeletal muscle much more efficiently than the analogous AAV2/2 vectors [6]. More recently, AAV7 and AAV8 were isolated from rhesus monkey heart, and AAV2/7 and AAV2/8 vectors demonstrated much higher transduction in skeletal muscle and liver, respectively, than the corresponding AAV2 vector [2]. An attribute of alternative AAV serotypes is a lack of neutralization by antibodies against AAV2; moreover, neutralizing antibodies against AAV2 were more frequent in human subjects than neutralizing antibodies against other serotypes, including AAV1, AAV7, and AAV8 [2,5,7,8].

Glycogen storage disease type II (GSD-II; Pompe disease; MIM 232300) causes death in infancy from cardiorespiratory failure related to an underlying hypertrophic, dilated cardiomyopathy [9]. The deficiency of acid α-glucosidase (GAA; acid maltase; EC 3.2.1.20) in GSD-II has been corrected by high-level enzyme replacement therapy (ERT) in GAA-tolerant animal models [10,11] and in a minority of subjects in clinical trials of ERT [12,13]. Gene therapy could provide long-term, beneficial replacement of GAA in GSD-II, if a depot organ adequately secreted GAA to drive mannose-6-phosphate receptor-mediated uptake in cardiac and skeletal muscle. Intravenous administration of adenovirus vectors encoding GAA previously demonstrated generalized correction of glycogen storage in the GAA-knockout (GAA-KO) mouse model [14,15], although glycogen gradually reac-

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cumulated in the months following vector administration [16]. The appearance of anti-GAA antibodies correlated with the disappearance of secreted 110-kDa human (h) GAA precursor from the plasma [16]. Similarly, GAA was expressed at high levels in skeletal muscle following intramuscular injection of a hybrid [E1$^-$, polymerase$^-$, preterminal protein$^-$] Ad-AAV vector encoding hGAA in neonatal GAA-KO mice and transiently detected in plasma following intravenous injection of the Ad-AAV vector in adult GAA-KO mice; however, anti-GAA antibodies were elicited by hGAA expression and secretion of hGAA was transient with that vector in immunocompetent GAA-KO mice [17,18].

An AAV2/1 vector corrected glycogen storage when injected intramuscularly in immunocompetent GAA-KO mice; however, the effect was observed only in the injected muscle [19]. We previously demonstrated the secretion and uptake of hGAA with both an AAV2/2 and an AAV2/6 vector in immunodeficient, GAA-KO/severe-combined immunodeficiency (SCID) mice [18]. The GAA-KO/SCID mice were bred to avoid the neutralizing antibody response to hGAA introduced with an AAV or Ad vector [16,18], and this strain of GSD-II mice did not form anti-GAA antibodies in response to an [E1$^-$, polymerase$^-$] Ad vector encoding hGAA [20]. In summary, AAV vectors had not provided systemic correction of glycogen storage in GAA-KO mice, due to low GAA expression or the presence of neutralizing antibodies against GAA.

We administered an AAV2/8 vector encoding hGAA to GAA-KO/SCID mice to evaluate the potential of AAV vector-mediated gene therapy in GSD-II. We analyzed GAA expression and glycogen content in the heart, diaphragm, and skeletal muscle 24 weeks following AAV2/8 vector administration. Endpoints included endurance during Rota-rod testing and reduction in the urinary glucose tetramer previously shown to be elevated in Pompe disease, Glcα1-6Glcα1-4Glcα1-4Glc (Glc_4) [21–24]. We now report the efficacy of an AAV2/8 vector in a prototypical lysosomal storage disorder.

RESULTS

Correction of GSD-II with an AAV2/8 Vector

We investigated the efficacy of AAV2/8 vectors in GSD-II by administering vectors to immunodeficient GAA-

TABLE 1: GAA activity and glycogen content 24 weeks following AAV2/8 vector administration

	Group[a]	GAA activity		Glycogen content	
		GAA[b] (mean ± SD)	Fold[c]	Glycogen[d] (mean ± SD)	Decrease[e]
Liver	AAV, XY	3700 ± 1000***	26	—	—
	AAV, XX	1800 ± 640**	13	—	—
	Control	3.0 ± 0.5	—	—	—
	Normal	140 ± 34	—	—	—
Heart	AAV, XY	6.8 ± 4.6[f]	0.3	0.03 ± 0.05****	99
	AAV, XX	34 ± 40[g]	1.5	0.05 ± 0.06***	98
	Control	1.6 ± 0.0	—	2.4 ± 0.3	—
	Normal	22 ± 7.9	—	0.04 ± 0.03	—
Diaphragm	AAV, XY	45 ± 36	7.3	0.08 ± 0.07*	85
	AAV, XX	7.5 ± 1.0	1.2	0.5 ± 0.12	6
	Control	1.4 ± 0.2		0.53 ± 0.30	—
	Normal	6.2 ± 2.7		0.01 ± 0.08	—
Quadriceps	AAV, XY	12 ± 0.5*	0.9	0.44 ± 0.04**	57
	AAV, XX	2.0 ± 0.5	0.2	0.93 ± 0.25	9
	Control	1.7 ± 0.1	—	1.02 ± 0.27	—
	Normal	13 ± 1.2	—	0.02 ± 0.01	—
Gastrocnemius	AAV, XY	12 ± 14	1.2	0.84 ± 0.30	48
	AAV, XX	2.1 ± 0.5	0.2	2.4 ± 2.7	0
	Control	3.3 ± 0.6	—	1.6 ± 0.5	—
	Normal	10 ± 1.8	—	0.0 ± 0.0	—

[a] Groups as follows: AAV, XY, AAV-CBGAApA-treated male GAA-KO/SCID ($n = 4$); AAV, XX, AAV-CBGAApA-treated female GAA-KO/SCID ($n = 3$, except for diaphragm GAA, where $n = 2$); Control, untreated, age-matched male GAA-KO/SCID ($n = 4$); Normal, C57BL/6 ($n = 5$).
[b] nmol/h/mg protein.
[c] Group mean/Normal group mean.
[d] mmol glucose/g protein.
[e] Reduction compared to Control group (%).
[f] Range 2.7–13.4.
[g] Range 6.6–81.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
**** $P < 0.0001$.

KO/SCID mice to avoid any effect of humoral immunity against introduced hGAA. We packaged an AAV vector containing the hybrid CMV enhancer and chicken β-actin promoter to drive hGAA, AAV-CBhGAApA [18], as AAV2/8 [2] and administered 10^{11} particles by intravenous injection to GAA-KO/SCID mice at 3 months of age. We analyzed tissue GAA activity and glycogen content 24 weeks later (Table 1). GAA activity was significantly increased in the liver, and glycogen content was significantly reduced in skeletal muscles of male GAA-KO/SCID mice following administration of the AAV2/8 vector (Table 1). Heart glycogen content was significantly reduced to near-normal levels for both male and female GAA-KO/SCID mice that received the AAV2/8 vector ($P < 10^{-4}$), reflecting the efficacy of hGAA expression with that vector (Table 1). Light microscopy of periodic acid–Schiff (PAS) stained histologic sections confirmed the reduction of glycogen and restoration of normal myofiber structure in heart, diaphragm, and skeletal muscle at 24 weeks following AAV2/8 administration (Fig. 1).

Higher hGAA Expression in Male GSD-II Mice with an AAV2/8 Vector

Upon GAA analysis in the liver at 24 weeks following AAV2/8 vector administration in GAA-KO/SCID mice, GAA activity was twofold increased in the liver of male GAA-KO/SCID mice versus females ($P < 0.04$). GAA activity was also more highly elevated in the diaphragm and the quadriceps in male mice (Table 1). Glycogen content was significantly reduced in the diaphragm and quadriceps of male mice following AAV2/8 vector administration compared to untreated, affected GAA-KO/SCID mice (Table 1). The reduction in glycogen content of the diaphragm and quadriceps in male mice reflected a sex-related increase in efficacy for male mice with the AAV2/8 vector.

Western blot analysis of tissues revealed the processed ~76- and ~67-kDa forms of hGAA in the liver of

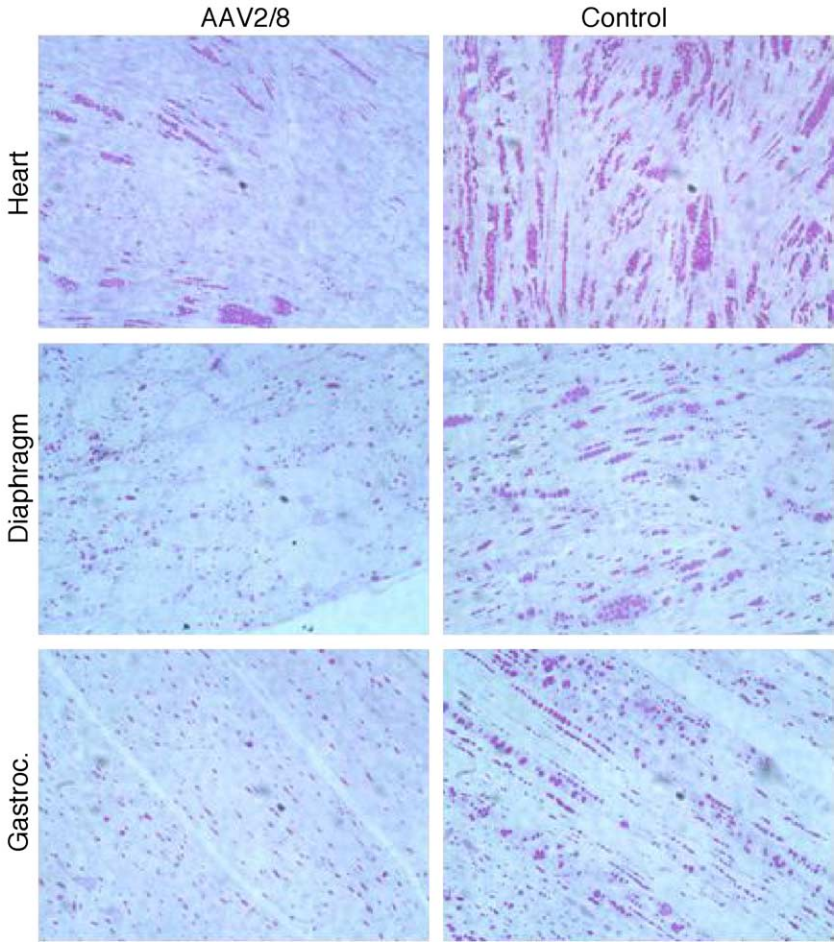

FIG. 1. Glycogen staining in muscle following AAV2/8 vector administration. PAS staining of glutaraldehyde-fixed, paraffin-embedded sections of gastrocnemius, diaphragm, and heart from a male GAA-KO/SCID mouse 24 weeks following AAV2/8 vector administration and from an age-matched, untreated male GAA-KO/SCID control.

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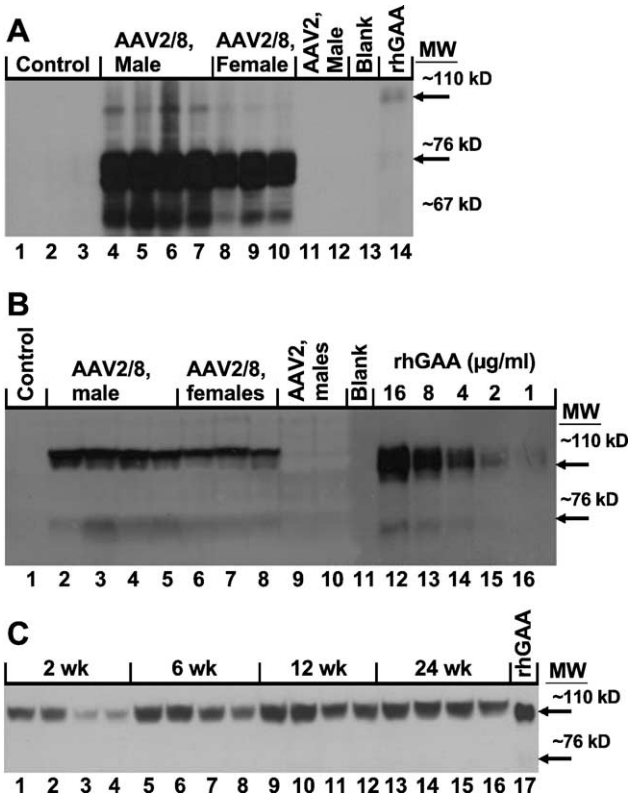

FIG. 2. Detection of hGAA following intravenous administration of an AAV2/8 vector in GAA-KO/SCID mice. Recombinant hGAA (rhGAA) was the standard. Each lane represents one mouse. (A) Western blot analysis of liver 24 weeks following vector administration. The ~67-, ~76-, and ~110-kDa hGAA species were detected in transduced liver [14,29]. (B) Western blot analysis of plasma. Samples were obtained 24 weeks following vector administration from treated mice and from age-matched, untreated GAA-KO/SCID controls. Each lane represents one mouse. Standards are shown for quantitation, and the concentration of rhGAA for each standard is indicated. 5 μl undiluted plasma was loaded for each sample. (C) Western blot analysis of plasma following AAV2/8 administration. Samples were obtained from male GAA-KO/SCID (n = 4) mice at 2, 6, 12, and 24 weeks following AAV2/8 vector administration and loaded in the same order for each time-point. 5 μl undiluted plasma was loaded for each sample.

GAA-KO/SCID mice following AAV2/8 vector administration (Fig. 2A). Male mice had detectable processed hGAA in the heart, diaphragm, and quadriceps muscle by Western blot analysis (not shown). In the liver the ~110-kDa hGAA precursor was present (Fig. 2A), and semiquantitative Western blotting revealed the presence of approximately 6 μg/ml hGAA of the ~110-kDa precursor form in the plasma of male mice with the AAV2/8 vector (Fig. 2B). The level of hGAA in plasma increased slightly between 2 and 6 weeks following vector administration, but it was sustained between 6 and 24 weeks postinjection (Fig. 2C). This level of hGAA in plasma was associated with approximately normal GAA activity and reduced glycogen content in heart, diaphragm, and quadriceps muscle of male GAA-KO/SCID mice (Table 1). The plasma hGAA level in female GAA-KO/SCID mice was approximately 50% of the level in males (Fig. 2B).

We evaluated the effects of the AAV serotype and an immune response to introduced hGAA on efficacy. When we injected 10^{11} particles of the AAV2/2 vector intravenously, no hGAA was detected by Western blotting of plasma 24 weeks postinjection (Fig. 2B, lanes 9 and 10). Similarly, when we administered 10^{11} particles of the

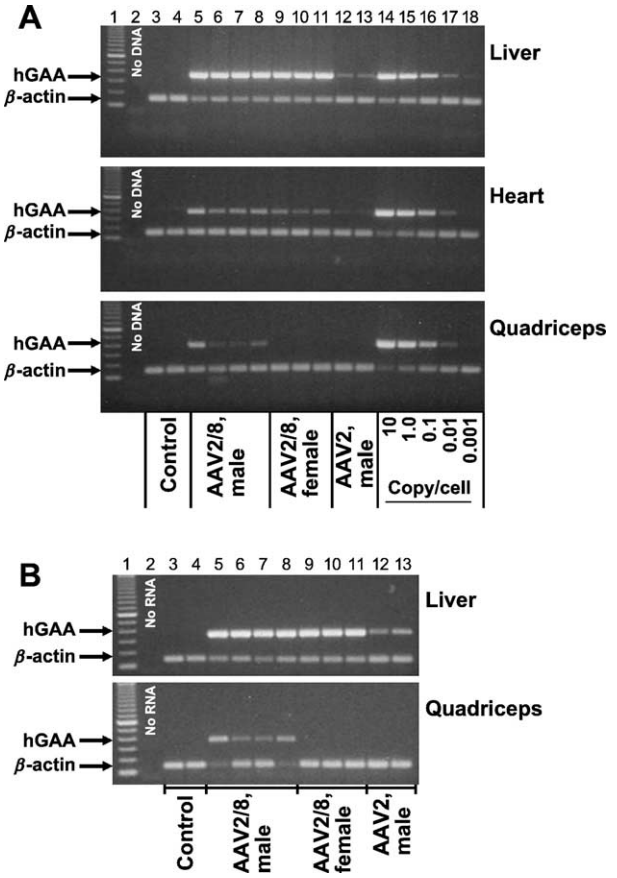

FIG. 3. Vector DNA analysis by semiquantitative PCR and vector RNA analysis by RT-PCR. (A) Semiquantitation of vector DNA by PCR for liver DNA, heart DNA, and quadriceps DNA 24 weeks following AAV2/8 vector administration for treated mice (n = 7; 4 males, lanes 5–8, and 3 females, lanes 9–11) and AAV2/2 (AAV2) vector administration for treated mice (n = 2 males; lanes 12 and 13) and for untreated, age-matched GAA-KO/SCID male controls (n = 2; lanes 3 and 4). Each lane represents an individual mouse. Lane 1 shows a 100 bp ladder molecular weight marker. The negative control consisted of no input DNA (lane 2). The control samples for quantitation consisted of added vector plasmid DNA representing from 10 to 0.001 copy/cell of AAV vector plasmid DNA in liver DNA from an untreated, GAA-KO/SCID mouse (lanes 14–18). (B) RT-PCR analysis of liver and quadriceps RNA at 24 weeks following AAV2/8 or AAV2 vector administration. Samples represent the same mice as in (A). The negative control consisted of no input RNA (lane 2). The control RNA from untreated GAA-KO mice revealed no hGAA signal (lanes 3 and 4). Mouse β-actin RNA was amplified as an internal control for each sample.

AAV2/8 vector to immunocompetent GAA-KO mice, hGAA was undetectable in plasma at 6–12 weeks post-injection (not shown). An ELISA of anti-GAA antibodies in the plasma of the immunocompetent GAA-KO mice done 6 weeks following AAV2/8 vector administration revealed titers of 1:1600 to 1:6400 (n = 3), whereas untreated GAA-KO mice had anti-GAA antibody titers <1:200 (n = 3).

Vector DNA quantitation in tissues demonstrated an AAV serotype-related difference in vector genome copy number at 24 weeks following AAV2/8 vector administration (Fig. 3A). Vector copy number for AAV2/8-treated GAA-KO/SCID mice was approximately 10 vector genomes/cell in the liver, which was >100-fold higher than the approximately 0.1 vector genome/cell for the AAV2/2 vector in liver (Fig. 3A, lanes 5–11 versus lanes 12 and 13). AAV vector genome copy number was approximately equivalent in the liver and heart of male mice compared to females (lanes 5–8 compared to lanes 9–11). AAV vector DNA was detected in quadriceps DNA only for male mice (lanes 5–8). Interestingly, hGAA expression with the AAV2/8 vector in the quadriceps muscle as detected by RT-PCR of vector RNA (Fig. 3B) was associated with the presence of AAV vector genomes at very low copy number (<0.1 vector genome/cell) (Fig. 3A). However, the presence of >0.001 vector genome/cell in the heart following intravenous AAV2/2 vector administration (Fig. 3B, lanes 12 and 13) was inadequate to drive expression of hGAA above the background activity measured in untreated, control GAA-KO/SCID mice (not shown).

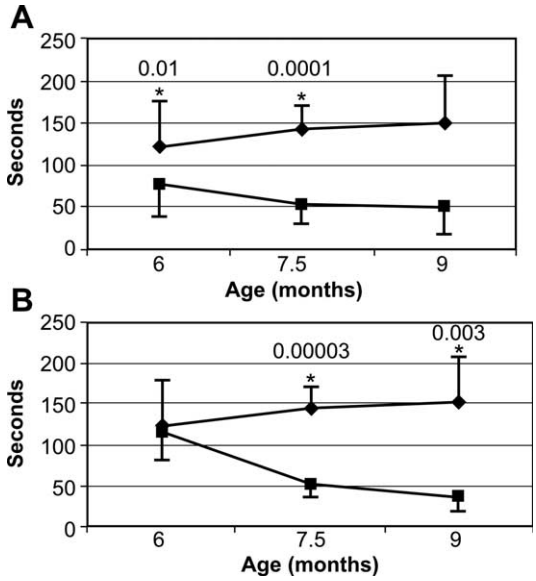

FIG. 4. Accelerating Rota-rod performance of GAA-KO/SCID mice. Experimental values significantly differing from control group values are marked (*), and the P values are indicated. The average Rota-rod time for each group of mice at the indicated ages is shown with the standard deviation. (A) Male GAA-KO/SCID mice following AAV2/8 administration (diamonds; n = 5 at 6 and 7.5 months, n = 4 at 9 months) and age-matched, untreated male GAA-KO/SCID controls (squares; n = 7 at 6 and 7.5 months, n = 6 at 9 months). (B) Female GAA-KO/SCID mice following AAV2/8 vector administration (diamonds; n = 3) and age-matched, untreated, female GAA-KO/SCID controls (squares; n = 3 at 6 and 7.5 months, n = 4 at 9 months).

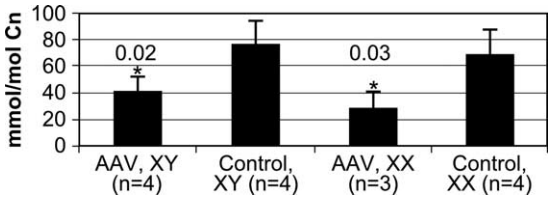

FIG. 5. Urinary Glc_4 following AAV2/8 vector administration. The average [Glc_4] is shown for the groups of GAA-KO/SCID mice 24 weeks following AAV2/8 vector administration and for age-matched, untreated GAA-KO/SCID mice. Male (XY) and female (XX) groups were analyzed separately. Data points significantly altered compared to controls are marked (*), and the P values are indicated. Cn, creatinine.

Improved Ambulation in GSD-II Mice

We used Rota-rod testing to demonstrate the improved endurance of GAA-KO/SCID mice following AAV2/8 vector administration (Fig. 4). Rota-rod time was significantly increased for male GAA-KO/SCID mice at 6 and 7.5 months of age (Fig. 4A) and significantly increased for pooled Rota-rod results of male and female GAA-KO/SCID mice following AAV2/8 vector administration at 6 ($P = 0.01$), 7.5 ($P < 10^{-4}$), and 9 months of age ($P < 10^{-2}$) compared to age-matched, untreated GAA-KO/SCID control mice. For female GAA-KO/SCID mice the AAV2/8 vector significantly increased Rota-rod times at 7.5 and 9 months of age compared to age-matched controls (Fig. 4B). Thus, the improved endurance of GSD-II mice was associated with decreased glycogen storage in the heart of both male and female mice following AAV2/8 vector administration.

Reduced Glucose Tetramer in GSD-II Mice

Glc_4 is a biomarker for GSD-II that reflects elevated glycogen storage in muscle. We analyzed urinary Glc_4 by tandem mass spectrometry in these groups of GAA-KO/SCID mice at 9 months of age (Fig. 5). The level of Glc_4 in urine was significantly reduced in male and female mice at 24 weeks after AAV2/8 vector administration compared to untreated, sex-matched controls. Reduced Glc_4 levels were associated with normalization of glycogen content in the heart of GSD-II mice following the introduction of hGAA with an AAV2/8 vector.

Discussion

AAV vectors have gained favor in gene therapy experiments due to low toxicity and long-term expression of introduced genes; however, the efficacy of AAV2/2 vectors has frequently been limited in mouse models for genetic disease by inefficient transduction in target

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tissues. The availability of alternative serotypes with improved tissue tropism has advanced gene therapy with AAV vectors. The initial characterization of an AAV2/8 vector reported 100-fold increased transduction of cells in the liver compared to an analogous AAV2/2 vector [2]. Here we report the efficacy of hGAA expressed with an AAV2/8 vector in a mouse model for GSD-II and demonstrate very high level GAA activity achieved in the depot organ, the liver. Normal GAA activity was achieved in the heart, diaphragm, and skeletal muscle following a single, intravenous injection of a modest number of AAV2/8 vector particles in male GSD-II mice. Indeed, following administration of an equivalent number of AAV2/8 or AAV2/2 vector particles, the vector genome copy number in liver with the AAV2/8 vector was >100-fold increased compared to the AAV2/2 vector. The current report and another describing efficacious gene therapy in hemophilia A mice [25] establish a role for AAV2/8 vectors in preclinical studies of gene therapy in animal models, especially with regard to the enhanced production of secreted therapeutic proteins in the liver. The basis for improved expression of introduced genes with AAV2/8 vectors was recently associated with rapid uncoating of vector genomes compared to AAV2/2 vectors [26].

Female GSD-II mice had a somewhat reduced response to the AAV2/8 vector compared to male mice, and glycogen content was significantly reduced only in the heart of female GSD-II mice, not in the diaphragm or quadriceps. A reduction in the conversion of single-stranded AAV vector genomes to double-stranded genomes was reported for AAV2/2 and AAV2/5 vectors in female mice, which was reversible by pretreatment with androgens [27]. The reduced transduction of liver in female mice was not associated with reduced AAV2 or 5 receptors in female mice, because equivalent numbers of single-stranded vector genomes were present in the nucleus for male and female mice. Although the AAV2/8 vector presented here demonstrated enhanced hGAA expression in male compared to female GAA-KO/SCID mice, it was efficacious in both male and female mice.

The data presented here support the hypothesis of secretion of hGAA from the liver as a depot and receptor-mediated uptake by heart and skeletal muscle, although low copy number vector DNA was present in the heart and skeletal muscle and some expression occurred at least in skeletal muscle. The ~110-kDa hGAA precursor is the precursor form that enters GAA-deficient cells through receptor-mediated uptake [10,28,29], and the presence of precursor hGAA in the liver and plasma was consistent with the hypothesis of hGAA secretion by the liver and uptake by other tissues. The presence of normal GAA activity in skeletal muscle suggested that uptake of hGAA from the plasma occurred, because the very low copy vector DNA in the quadriceps of male AAV2/8-treated GAA-KO/SCID mice would be inadequate to reduce the glycogen content of the entire muscle in the absence of cross-correction through hGAA secretion and uptake. The presence of very low level hGAA expression in the quadriceps as detected by RT-PCR demonstrated only that the vector DNA was transcribed in a minority of cells that were transduced. The hypothesis that the liver was converted to a depot for hGAA production is consistent with the mechanism of ERT in GSD-II, in which the hGAA precursor is taken up efficiently from the bloodstream.

Efforts to develop gene therapy in GSD-II are justified by the limitations of ERT for GSD-II, which include the need for frequent infusions of high-level doses of GAA replacement to achieve efficacy. High-level hGAA replacement (40–100 mg/kg/dose) has reduced the glycogen content of heart and skeletal muscle in GAA-KO mouse models [11,30], and hGAA doses from 5 to 40 mg/kg/week improved outcome measures in some Pompe infants enrolled in clinical trials of ERT [13,31]. The amount of hGAA required to achieve efficacy is approximately 10- to 100-fold higher than the doses for ERT in other lysosomal disorders [32], and the potential cost of such high-level enzyme production provides justification for attempts to develop gene therapy in GSD-II. Efficacious replacement of hGAA in a GSD-II mouse model persisted for 24 weeks following a single, intravenous injection of the AAV2/8 vector encoding hGAA, which compares favorably with ERT for GSD-II in terms of frequency of treatment and ease of production.

The formation of anti-GAA antibodies and associated infusion reactions prevented continuation of ERT beyond 3 weeks in nontolerant GAA-KO mice [11]. Only by the generation of tolerant GAA-KO mice by insertion of a low-expressing liver-specific transgene could long-term ERT be tested in a GSD-II mouse, and a reduction in the glycogen content of skeletal muscle required administration of 100 mg/kg recombinant hGAA (a high dose compared to other forms of ERT) [11]. We previously administered an AAV2 vector encoding hGAA in immunocompetent GAA-KO mice and documented antibody formation 6 weeks postinjection [18]. Similarly, the AAV2/8 vector described here induced neutralizing anti-GAA antibody formation in immunocompetent GAA-KO mice. Development of an AAV2/8 vector encoding hGAA in immunodeficient GAA-KO mice is important for establishing that efficacy can be achieved with an AAV vector in GSD-II. Furthermore, many GSD-II patients synthesize residual, partially active GAA protein, and therefore a subset of GSD-II patients is likely to be tolerant to GAA introduced by gene therapy [9].

Appropriate endpoints are critical to the design of clinical trials, especially in rare diseases with small patient populations in which the use of placebo controls might be deemed unethical. A trial of gene therapy in infantile GSD-II (Pompe disease) could present problems in this light, since prolonged survival in the treatment group versus a placebo-control group would be an

unacceptable endpoint. Therefore, we evaluated two surrogate outcome measures for gene therapy in the mouse model for GSD-II, the Rota-rod test and the Glc_4 urinary oligosaccharide. The Rota-rod test is similar to timed, functional tests of muscular abilities that could thus prove useful as outcome measures in clinical trials of gene therapy for GSD-II (especially for adult-onset GSD-II with preserved ambulation), as has been demonstrated during natural history studies in Duchenne muscular dystrophy [33]. Furthermore, the Glc_4 urinary oligosaccharide serves as a biomarker for GSD-II [24,34], and we demonstrated here that reduced urinary Glc_4 was associated with reduced heart glycogen content following gene therapy.

Therapeutically relevant levels of human protein synthesis have been achieved with AAV vectors in several animal models for genetic disorders, including lysosomal storage diseases, Duchenne muscle dystrophy, and hemophilia [35–43]. These developments had implications for gene therapy in GSD-II, because GSD-II is both a lysosomal storage disorder and a muscular dystrophy [9,44]; moreover, gene therapy in hemophilia established the model of a depot organ for the production of a therapeutic protein, as has been proposed for gene therapy in GSD-II [14,15,36–41]. However, AAV vectors have not previously been efficacious in GSD-II mice [18,19], possibly related to the high level of hGAA needed to correct muscle GAA deficiency in GSD-II [11,13,30,31] or to the high likelihood of eliciting neutralizing anti-GAA antibodies in GAA-KO mice [16,18]. The comparison of AAV2/2 and AAV2/8 vector encoding hGAA presented here demonstrated that cross-packaging the AAV vector as AAV8 exceeded the therapeutic threshold for hGAA production, albeit in the absence of a complicating immune response.

The presence of neutralizing antibodies against GAA in GAA-KO mice following enzyme replacement or gene therapy remains an obstacle to efficacious therapy [16,45]. GSD-II patients with no residual GAA protein could have a diminished response to enzyme replacement therapy or gene therapy [13], although the majority of GSD-II patients synthesize residual, inactive GAA and could be expected to respond to sustained, therapeutic hGAA expression with an AAV2/8 vector [9,44].

Materials and Methods

Cell culture. 293 cells and C-7 cells [46] were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U penicillin per milliliter, and 100 μg streptomycin per milliliter at 37°C in a 5% CO_2–air atmosphere. C-7 cells were grown in the presence of hygromycin, 50 μg/ml.

Preparation of AAV vectors. AAV2/2 vector stocks were prepared as described with modifications [18]. Briefly, 293 cells were transduced with the hybrid Ad-AAV vector (2000 DNase I-resistant vector particles/cell as quantitated by Southern blot analysis) containing the AAV vector genome 15–30 min before transfection with AAV packaging plasmids containing the AAV2 Rep (pMTRep) and Cap (pCMVCap) genes driven by heterologous promoters, which typically generate no detectable replication-competent AAV (rcAAV) [47]. The hybrid [E1$^-$, polymerase$^-$, preterminal protein$^-$] Ad-AAV vector was deleted for Ad polymerase, and therefore it does not replicate in 293 cells to generate contaminating Ad particles [18]. Cell lysate was harvested 48 h following infection, freeze–thawed three times, and isolated by iodixanol step gradient centrifugation before heparin-affinity column purification [48]. For AAV2/8 vector stocks, the AAV packaging plasmid was p5E18-VD 2/8 [2] (courtesy of Dr. James M. Wilson, University of Pennsylvania, Philadelphia, PA, USA). AAV2/8 vector stocks were purified as described [49] by sucrose cushion pelleting followed by two cesium chloride gradient centrifugation steps. AAV stocks were dialyzed against three changes of Hanks' buffer, and aliquots were stored at −80°C. The number of vector DNA-containing-particles was determined by DNase I digestion, DNA extraction, and Southern blot analysis. All viral vector stocks were handled according to Biohazard Safety Level 2 guidelines published by the NIH.

In vivo administration of AAV vector stocks. The AAV vector stocks were administered intravenously (via the retro-orbital sinus) in 12-week-old GAA-KO/SCID mice [20], which were generated by crossing SCID and GAA-KO mice [50]. The immunodeficient SCID mice have a spontaneous point mutation in the catalytic subunit of the DNA-dependent protein kinase [51] gene and lack both B- and T-cell-mediated immunity [52,53]. At the indicated time points postinjection, plasma or tissue samples were obtained and processed as described below. All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

Determination of hGAA activity. hGAA activity was measured following removal of tissues from control or treated mice, flash-freezing on dry ice, homogenization and sonication in distilled water, and pelleting of insoluble membranes/proteins by centrifugation. The protein concentrations of the clarified suspensions were quantified via the Bradford assay. hGAA activity tissues were determined as described [14].

Glycogen content of tissues was measured using the *Aspergillus niger* assay system, as described [54]. A two-tailed homoscedastic Student *t* test was used to determine significant differences in hGAA levels, glycogen content, and other measurements between GAA-KO mice with or without administration of the vector encoding hGAA.

Western blotting analysis of hGAA. For direct detection of hGAA in tissue homogenates, 50 μg of total protein for each sample [55] was electrophoresed overnight in a 6% polyacrylamide gel to separate proteins and transferred to a nitrocellulose membrane. The blots were blocked with 5% nonfat milk solution, incubated with primary and secondary antibodies, and visualized via the enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ, USA) [55]. For Western blotting analysis of hGAA in plasma, 5 μl of undiluted plasma was loaded for each sample.

Semiquantitation of AAV vector DNA by PCR. Genomic DNA was extracted from GAA-KO/SCID mouse tissues, and PCR was performed in a 50-μl reaction containing 500 ng of mouse DNA, 2.5 units of *Taq* DNA polymerase with 1× PCR buffer (Qiagen, Valencia, CA, USA), and 150 ng each of the sense and antisense primers. Gene-specific primers for hGAA (sense, 5′-AGTGCCCACACAGTGCGACGT-3′, nucleotide 672 to 692, and antisense, 5′-CCTCGTAGCGCCTGTTAGCTG-3′, nucleotide 998 to 1018; GenBank NM 000152) and for mouse β-actin (sense, 5′-AGAGG-GAAATCGTGCGTGAC-3′, and antisense, 5′-CAATAGTGATGACCT-GGCCGT-3′ [56]) were used for each reaction. Samples were denatured at 94°C for 3 min, followed by 32 cycles (27 cycles for β-actin, internal control) of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. Plasmid DNA corresponding to 0.001 to 10 copies of human GAA cDNA per cell (in 500 ng genomic DNA) was mixed with 500 ng of genomic DNA from control (mock) GAA-KO/SCID mouse as the standards for semiquantitative assay. The reaction was terminated with a 10-min extension at 72°C. Aliquots of 20 μl of each PCR were electrophoretically separated on 1.2% agarose gel with ethidium bromide and photographed.

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RT-PCR. Three micrograms of total RNA isolated from liver or quadriceps was DNase I treated and subsequently reverse-transcribed with 300 units of M-MLV reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, USA) and 300 ng of random hexamer primers in a 40-μl reaction. Four microliters of cDNA was subjected to PCR as described above. Samples were denatured at 94°C for 3 min, followed by 32 cycles (27 cycles for β-actin, internal control) of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. Primers for RT-PCR were identical to those used in semiquantitation of AAV vector DNA.

Rota-rod performance test. All mice were tested at different time points on a Rota-rod device (Ugo Basile, Italy). Each mouse was conditioned to the device by performance of two 30-s attempts on the rod at a constant speed of 4 rpm. Following the conditioning phase, each mouse was then placed on the rod and timed for its ability to remain on the rod as it accelerated to a maximal rate of 40 rpm. This was repeated and the average of the runs was used as the final endurance time for each mouse. At each time point the average performance of all mice at each time point was plotted against time post-vector injection, and the standard deviation was calculated for each time point. Two-tailed homoscedastic Student *t* tests were utilized to determine the significant difference in accelerating Rota-rod performance.

Urinary Glc_4. Urinary Glc_4 concentrations were determined relative to creatinine by stable isotope-dilution electrospray tandem mass spectrometry as previously described [34].

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